AD_____

Award Number DAMD17-97-1-7317

TITLE: Functional Analysis of Breast Cancer Susceptibility Gene BRCA2

PRINCIPAL INVESTIGATOR: Lihua Y. Marmorstein, Ph.D.

CONTRACTING ORGANIZATION: Mount Sinai School of Medicine
New York, New York 10029

REPORT DATE: November 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. AGENCY USE ONLY *(Leave blank)* | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | November 1998 | Annual (11 Jul 97 - 11 Jul 98) |

**4. TITLE AND SUBTITLE**
Functional Analysis of Breast Cancer Susceptibility Gene BRCA2

**5. FUNDING NUMBERS**
DAMD17-97-1-7317

**6. AUTHOR(S)**
Lihua Y. Marmorstein, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Mount Sinai School of Medicine
New York, New York 10029

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200 words)*

In order to study the physiological function and the mechanism by which the breast cancer susceptibility gene BRCA2 is involved in breast cancer, I have generated and characterized a series of polyclonal and monoclonal antibodies against BRCA2. By using these antibodies, I demonstrated that the *BRCA2* gene product is a 460-kDa nuclear phosphoprotein, and that abnormality in its distribution does not seem to be the mechanism by which it is involved in tumorigenesis. I also found that BRCA2 forms in vivo complexes with both p53 and RAD51. Moreover, exogenous BRCA2 expression in cancer cells inhibits p53's transcriptional activity, and RAD51 co-expression enhances BRCA2's inhibitory effects. These findings demonstrate that BRCA2 physically and functionally interacts with two key components of cell cycle control and DNA repair pathways. Thus, BRCA2 likely participates with p53 and RAD51 in maintaining genome integrity. The major goals of this study are to provide a basis for designing agents that could regulate BRCA2's production or replace its function by the molecular dissection of its functional pathways, and to identify components of the signaling pathway of BRCA2 that may be used as novel biomarkers for breast tumor prognosis, with the goal of developing effective intervention protocols.

**14. SUBJECT TERMS**
Breast Cancer

BRCA2, mutation, tumor suppressor gene, subcellular localization, tumorigenesis

**15. NUMBER OF PAGES**
29

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

## FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

_____ Where copyrighted material is quoted, permission has been obtained to use such material.

_____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

_____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

_____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

L.Y.M. ✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

L.Y.M. ✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

L.Y.M. ✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

L.Y.M. ✓ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Yao Chen 11/11/98

PI - Signature Date

# Table of Contents

# Introduction

The study of cancer has been documented to have originated at least 3600 years ago, yet today there is still no way to put this group of diseases completely under control. The high incidence of breast cancers among women in Western societies has prompted intensive research on this disease. Important breakthroughs came with the identification of the breast cancer susceptibility genes BRCA1 and BRCA2, which confer susceptibility to early-onset familial breast and ovarian cancers (1-4). Inherited mutations in BRCA1 and BRCA2 are believed to be responsible for most hereditary breast cancers (3-10), which account for 5-10% of all breast cancer cases (11). About 52% of the families with four or more breast cancer cases have inherited mutations in BRCA1, and 32% possess BRCA2 mutations (12). In contrast, somatic mutations in either of these two genes are very rare in sporadic breast cancers (5, 13-15), which account for the remaining 90-95% of all breast cancers (11).

Although mutation screening of BRCA1 and BRCA2 give researchers the ability to identify cancer risk in some individuals, they do not offer satisfactory intervention or treatment for these individuals. The mutation studies suggest that if BRCA1 and BRCA2 play significant roles in tumorigenesis in non-hereditary breast cancers, structural mutations of the genes themselves are not involved. Therefore, to elucidate the mechanisms by which BRCA1 and BRCA2 are involved in cancer, functional studies on BRCA1 and BRCA2 are of paramount importance.

Both BRCA1 and BRCA2 genes encode large, hydrophilic proteins. Both genes are up-regulated in proliferating and differentiating cells, expressed in a cell cycle-dependent manner peaking at the G1/S boundary (ref.19-21 and my unpublished data), widely expressed during development (22-24), and essential for early embryonic development (25-29). Recent evidence indicates that BRCA1 is involved in transcriptional regulation (30, 31). The BRCA2 gene is composed of 27 exons and encodes a predicted 384-kDa protein possessing no obvious homology to other sequences in publicly available databases (4). There are eight repetitive units in the BRCA2 protein sequence (BRC motifs, ref. 16 and 17) and a potential nuclear localization signal within its C terminus (18). BRCA2 also possesses a putative transcriptional activation domain in exon 3, suggesting a role of BRCA2 in the regulation of gene expression (32). Evidence for a possible function of BRCA2 in DNA repair has been suggested by recent findings that BRCA2 mutant mouse embryos are defective in DNA repair (27, 33, 34). Moreover, yeast two-hybrid and glutathione S-transferase (GST) pull-down analysis revealed that BRCA2 interacts in vitro with RAD51, a protein involved in DNA double-strand break repair and homologous recombination (ref. 27, 35, 36 and my unpublished data). Binding sites for RAD51 have been mapped to each of the eight BRC motifs in human BRCA2 (36) and to a C-terminal region of mouse BRCA2 (27, 35).

Structural and functional characterization of the endogenous BRCA2 protein have been hampered by the large size of the protein and the lack of suitable immunological reagents for its detection. To this end I have generated and characterized five polyclonal and three monoclonal antibodies against BRCA2. Two of the polyclonal antibodies were generated against peptides at the N-terminus (N-19) and C-terminus (C-15) of BRCA2, and three other polyclonal and all the monoclonal antibodies were generated against GST-fusion proteins corresponding to regions close to the N-terminus (GSTB1), middle (GSTB2) and C-terminus (GSTB3) of BRCA2. By using these antibodies, I have demonstrated that the BRCA2 gene product is a 460-kDa nuclear phosphoprotein that associates in vivo with a significant portion of the endogenous pool of RAD51. Because an immediate cellular response to DNA damage is p53-mediated cell cycle arrest (37), and RAD51 has been reported to physically associate with p53 (38, 39), I also investigated whether a physical and functional relationship could be detected between BRCA2 and p53. The identification of molecules interacting with BRCA2 provides clues as to how BRCA2 is modified, and in what signal transduction pathways BRCA2 may be involved.

# Body of the Report

## Experimental Methods

**Generation and characterization of antibodies against BRCA2** Five polyclonal and three monoclonal antibodies were generated. Two of the polyclonal antibodies were generated against peptides at the N-terminus (N-19) and C-terminus (C-15) of BRCA2, and three other polyclonal and all the monoclonal antibodies were generated against GST-fusion proteins corresponding to regions close to the N-terminus (GSTB1), middle (GSTB2) and C-terminus (GSTB3) of BRCA2. The peptides were conjugated to keyhole limpet hemacyanin. Three different regions of BRCA2 were amplified by reverse transcription followed by polymerase chain reaction (RT-PCR) from the normal breast epithelial cell line AB5. PCR primers were synthesized according to the published BRCA2 sequence. Restriction enzyme sites were incorporated into primers by appropriate mutations. For region 1 (GSTB1), upstream primer is 5'-GGTCAAGTTCTTTAGCTACAGGATCCACCC-3' containing a BamHI site; downstream primer is 5'-CTCCATCTGGGCTCCATGTCGACCTGAAAG-3' containing a SalI site. For region 2 (GSTB2), upstream primer is 5'-CCTGCAACTTGTTACAGAATTCAGTCCCC-3' containing a EcoRI site; downstream primer is 5'-AGGGTGAAGAGCTAGTCTCGAGTTCCTCAA-3' containing a XhoI site. For region 3 (GSTB3), upstream primer is 5'-GTCAGTGAATCCACTAGAATTCCTCCCACC-3' containing a EcoRI site; downstream primer is 5'-TGGTCTTGAACTCCTGGCCTCGAGCACT-3' containing a XhoI site. GSTB1 is a 647-bp fragment from codons 194 to 409, GSTB2 a 508-bp fragment from codons 1651 to 1820, and GSTB3 a 500-bp fragment from codons 3365 to 3418. The PCR products were purified by 1% agarose gel electrophoresis, cut with restriction enzymes, purified again and subcloned into the GST-fusion protein expression vector pGEX-5X-2 or pGEX-4T-2 (Pharmacia,) to generate GST-BRCA2 proteins. The GST-BRCA2 fusion constructs were verified by sequencing using 5' and 3' pGEX sequencing primers (Pharmacia). The GST-fusion proteins were expressed in DH10B bacterial cells (GIBCO) and purified by affinity chromatography with glutathione sepharose 4B. Two fusion proteins (region 1 and 3) were further purified by 12% SDS-polyacrylamide gels (SDS-PAGE). All antibodies were affinity-purified.

**Immunoprecipitation** Cells were lysed in lysis buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl [PMSF], and 1 mM sodium orthovanadate). The total protein content of the lysates was determined by the BCA assay (Pierce). In some cases, cells were labeled with [$^{35}$S]methionine (0.1 mCi/ml) or [$^{32}$P]phosphoric acid (0.25 mCi/ml; NEN) for 3 hr. Phosphatase treatment of immunoprecipitates was performed as described (40). Protein was precipitated with the corresponding antibody for 3 hr by rocking at 4°C. Precipitates were washed four times with lysis buffer, and then electrophoresed on 4-10% SDS-PAGE gels. Rainbow prestained standards (Amersham) were co-electrophoresed on adjacent lanes.

**Immunoblotting** After electrophoresis, proteins were electrophoretically transferred from the gel onto a poly(vinylidene difluoride) (PVDF) membrane (Millipore). The residual binding sites on the membrane were blocked by 1% milk in PBS containing 0.1% Tween 20 for 1 hour at room temperature. The blots were then incubated for 1 hr at room temperature with a primary antibody. After removing the primary antibody the blots were washed 3 times (10 min each), and then incubated for 1 hr with a secondary antibody. ECL or ECLPlus (Amersham) was used for signal detection. Anti-GFP antibody was obtained from CLONTECH, antibodies against p53 (PAb421), DNA-dependent protein kinase, or RAD51 from Calbiochem, antibodies against the HA epitope or p53 (DO-1) from Santa Cruz Biotechnology, and anti-retinoblastoma protein (RB) antibody from PharMingen.

**Plasmid constructs** pGFPB2 containing human full-length BRCA2 cDNA fused in-frame with green fluorescent protein (GFP) was constructed as follows. A fragment at the n-terminus of BRCA2 was generated from pUCBRCA2 by PCR amplification using a high-fidelity PCR system (Boehringer Mennheim) and the following primers: 5'-GGCCAGATCTATGCCTATTGGATCCAAAGAGAGG-3' and 5'-GGCCGTCGACTGCTTGTTTATCACCTGTGTCTCC-3'. This fragment was subcloned into the pEGFPC1 vector (CLONTECH) at BglII and SalI sites to generate pGFPB2N. A Sse8387I-SalI BRCA2 fragment released from pUCBRCA2 (Myriad) then was ligated with pGFPB2N digested with BglII and Sse8387I to generate pGFPB2. Sequencing confirmed the identity of the fragment generated by PCR. A human full-length RAD51 cDNA with BamHI and SalI flanking was amplified from a human testis cDNA library (CLONTECH) by using Pfu DNA polymerase (Stratagene) and the following primers: 5'-GGCCGGATCCATGGCAATGCAGATGCAGCTTG-3' and 5'-GGCCGTCGACTCTTTGGCATCT CCCACTCCAT-3'. Cloning of this cDNA fragment into $pcDNA_3HA$ generated an N-terminal hemagglutinin (HA) tagged human RAD51 construct, pHA-RAD51. A BglII-BamHI fragment containing two copies of the consensus p53 binding sequence and the adenovirus major late TATA box from p50-2 were cloned into the pGL2 vector (Promega) to generate a luciferase reporter plasmid PGluc. GAL4luc and GAL4-VP16 were a gift from R. Davis (Univ. of Massachusetts, Worcester).

**Immunofluoresence** Cells were grown to 70% confluency on glass cover slips in 6-well tissue culture plates, then washed with phosphate buffered saline (PBS), fixed for 30 minutes in 2% paraformaldehyde in PBS, or 20 minutes in chilled methanol. After blocking in PBS containing 2% BSA, Cells were stained with a primary antibody for 1 hour at 37°C, washed 3 times, and then incubated with a secondary antibody conjugated to fluorescein, rhodomine or CY3 (Rockland) for 1 hour. Cells were washed and, in some cases, counterstained with DAPI to label nuclei. After a final wash coverslips were mounted in vectashield (Vector Labs) and sealed with nail polish. Labeled cells were visualized with a Nikon epifluorescence microscope. To detect the localization of exogenous BRCA2, pGFPB2 was transiently transfected into 293T cells in 6-well plates containing coverslips using Lipofectamine (GIBCO). Twenty-four hours after transfection, cells were incubated with hoechst stain (No. 33258; 5 μg/ml; Sigma) for 15 min to label nuclei. Coverslips were transferred to a Sykes-Moore perfusion chamber (Belco) mounted on the stage of a Nikon Eclipse E600 microscope and maintained at 37°C. Images were collected by using a Nikon Plan Apo X100 oil immersion objective lens (numerical aperture-1.4), a 1,000 X 800 pixel back-illuminated cooled charge-coupled device camera (Princeton Instruments), and metamorph software (Universal Imaging).

**Northern blot** Total RNA was isolated from cell lines with RNAzol. 10 μg of total RNA is denatured in 24.5 μl denaturing buffer. The denaturing buffer contains 2.2 M fomaldehyde, 1x 3-(N-morpholino) propane sulfonic acid (MOPS, 5 mM sodium acetate, 0.1 mM EDTA), and 50% formamide. RNA samples were then heated for 10 minutes at 60°C, mixed with 0.5 μl of 3% bromophenol blue, and electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde. After electrophoresis, the gel was rinsed briefly 3 times with water, followed by 50 mM NaOH, and neutralized by incubating in 1 M ammonium acetate twice. RNA samples were then transferred to nitrocellulose membranes in 1 M ammonium acetate. Following transfer, the membrane was washed in 1 M ammonium acetate for 30 minutes, air-dried, and baked at 80°C for 3 hours. The membrane was hybridized overnight at 42°C with a $^{32}P$-labelled DNA probe for BRCA2 as described previously (Kraus and Aaronson, 1993). The BRCA2 probes were the same RT-PCR products used to generate GST-fusion proteins, and were labeled with a nick translation kit (Amersham).

**Yeast two hybrid system for identifying proteins potentially interacting with BRCA2** A yeast two hybrid system (MATCHMAKER Two-Hybrid System 2) was obtained from Clontech (Palo Alto). BRCA2 fragments were amplified by RT-PCR from the normal breast epithelial cell line AB5. For the C-terminal region, upstream primer is 5'-CAGTTGGAAATTAGGAAGGCCATGGAATCTGCT GAA-3' containing a NcoI site; downstream primer is 5'-GGCCGTCGACTACCAATACGGAATCG

GGCAAAAC-3' containing a SalI site. For exon 11 region, upstream primer is 5'-CAAGAACTCTACC ATGGTTTTATATGGAGACACAGG-3' containing a NcoI site; downstream primer is 5'-GGCCGT CGACCTCAACAAGTGAGACTTTGGTTCC-3' containing a SalI site. The target protein vectors were constructed by inserting BRCA2 fragments in the correct reading frame and orientation into pAS2-1 to generate a hybrid of GAL4 DNA-BD and BRCA2. The fusion constructs were verified by restriction digestion and sequencing using the GAL4 DNA-BD junction primer. After transformation of the DNA-BD/BRCA2 plasmid into the reporter yeast strain CG-1945 using the YEASTMAKER yeast transformation system (Clontech), the expression and stability of the hybrid proteins were assayed by Western blot analysis using a GAL4 DNA-BD monoclonal antibody (Upstate Biotechnology). A human testis cDNA library and a human placenta cDNA library (Clontech) were used for screening genes potentially interacting with BRCA2.

## Results

**Characterization of the BRCA2 gene product.** To facilitate the study of BRCA2 structure and function, I generated a series of polyclonal and monoclonal antibodies using both peptides and GST fusion proteins corresponding to various regions of the molecule (Fig. 1A). On western blots of 293T cell lysates, affinity-purified polyclonal antibodies against both the N-terminal (anti-N19) and C-terminal peptides (anti-C15) recognized a band at a molecular mass much greater than that of the 220 kDa myosin standard (Fig. 1B). This band was also detected in MCF7 cells (Fig. 1 C, lane 3), and co-migrated with 460-kDa DNA-PK (Fig. 1C, lane 9). However, it was not detected by anti-C15 in CAPAN-1 cells (Fig. 1C, lane 4), a human pancreatic tumor cell line which has lost one *BRCA2* allele and contains the 6174delT mutation in the remaining allele (Table 1 and ref. 41).

To confirm that this band reflected the authentic BRCA2 protein, I transfected 293T cells with the plasmid pGFPB2 containing full-length *BRCA2* cDNA fused at its N-terminus to GFP, or with the control vector pEGFPC1. Both anti-N19 and anti-C15 specifically recognized a band in pGFPB2 transfected but not in vector transfected 293T cells at the predicted size of the GFP-BRCA2 fusion protein, approximately 27 kDa higher in mass than the native BRCA2 protein (Fig. 1B, lanes 1 and 5). A protein of the same size was also recognized by a polyclonal antibody against GFP (Fig. 1B, lane 9), confirming its identity as GFP-BRCA2. Moreover, all of these bands were specifically competed by the homologous peptides (Fig. 1B). Further confirmation that the proteins I had identified represent the authentic BRCA2 protein was provided by immunoprecipitation/western blot experiments. MCF7, CAPAN-1 or transfected 293T cell lysates were immunoprecipitated with a monoclonal antibody (mAb B2) raised against the GSTB2 region of BRCA2 (Fig. 1A), and then immunoblotted with anti-C15. The expected 460-kDa BRCA2 protein was detected in MCF7 and 293T cells but not in CAPAN-1 cells, and the 487 kDa GFP-BRCA2 fusion protein was only detected in pGFPB2 transfected 293T cells (Fig. 1C). All of these findings firmly established the identity of 460-kDa protein as the product of the *BRCA2* gene.

When BRCA2 was immunoprecipitated with mAb B2 from CAPAN-1 or MCF cell lysates, and immunoblotted with anti-N19, a truncated BRCA2 protein was specifically detected in CAPAN-1 cells (Fig. 1D, lane 1). This truncated species migrated above the 220 kDa myosin standard, and had an estimated molecular mass of 230 kDa, matching the predicted size of 224 kDa. These results indicate that the mutated *BRCA2* gene in CAPAN-1 cells encodes a stable truncated protein.

It should be noted that both anti-C15 and mAb B2 recognized a BRCA2 doublet in MCF7 and 293T cells (Fig. 1B and C), suggesting the possibility of post-translational processing, such as phosphorylation. Evidence that BRCA2 was phosphorylated derived from [$^{32}$P] labeling experiments, in which a [$^{32}$P] labeled band at 460 kDa was detected in autoradiographs of mAb B2 immunoprecipitates of MCF7, but not CAPAN-1 cells (Fig. 1E). However, when mAb B2 immunoprecipitates from MCF7 cells were treated with lambda-phosphatase, the BRCA2 doublet was not altered. Under the same conditions, the

retinoblastoma protein (RB) doublet, which is known to result from phosphorylation (42), was reduced to a single band (Fig. 1F). Thus, phosphorylation was unlikely account for the BRCA2 doublet. It should also be noted that anti-N19 only recognized the upper band of the BRCA2 doublet, and that GFP-BRCA2 migrated as a single band as well (Fig. 1B and C). Thus, the doublet likely resulted from BRCA2 amino-terminal processing or as an alternative product of the *BRCA2* gene.

**Exogenously expressed BRCA2 localizes to the nucleus.** BRCA2 reportedly possesses a potential nuclear localization signal in its C-terminus (18), and recently Bertwistle et al. (43) reported that BRCA2 was enriched in the nuclear fraction derived from MCF7 cells. While my antibodies recognized GFP-BRCA2 overexpressed in transfected cells, it was not possible to reliably detect endogenous BRCA2 by standard immunofluorescent methods (my unpublished data). However, the availability of the GFP tagged *BRCA2* construct made it possible to localize the transfected GFP-BRCA2 protein in live 293T cells by fluorescence microscopy. While GFP alone was distributed throughout the cell (data not shown), GFP-BRCA2 was primarily localized to the Hoechst labeled nuclei (Fig. 2). These results suggest a nuclear localization of the BRCA2 protein.

**Expression of BRCA2 in normal and cancer cells.** To gain information about whether BRCA2's transcription and translation are altered in cancer cells, how BRCA2's expression correlates with cell cycle progression, and whether there is a difference in BRCA2's expression during a specific cell cycle phase in tumor cells, I examined BRCA2's expression at both mRNA and protein levels in normal and cancer cells. An approximately 11-kb mRNA species of BRCA2 and the 460-kDa BRCA2 protein was found in all the cell lines examined except CAPAN-1 (Table 1). The expression level of BRCA2 is different in different cells, and peaks at G1/S cell cycle phase both in normal and cancer cells. These results indicate that BRCA2 is widely expressed both in normal and cancer cells.

**In vivo interaction of BRCA2 with RAD51.** BRCA2 has been reported to interact with RAD51 in vitro (refs 27, 35, 36 and my unpublished data). To investigate whether these proteins form a complex in vivo, BRCA2 was immunoprecipitated with mAb B2 from MCF7 or CAPAN-1 cell lysates, and immunoblotted with anti-RAD51. As shown in Fig. 3A, endogenous RAD51 was readily detected in a complex with endogenous BRCA2 in MCF7 cells (Fig. 3A, lane 4), while there was a faint signal for RAD51 co-immunoprecipitated with the truncated BRCA2 protein in CAPAN-1 cells (Fig. 3A, lane 3). As a specificity control, anti-IgG failed to immunoprecipitate complexes containing RAD51 from MCF7 cells (Fig. 3A, lane 5). The signal intensities for RAD51 in 50 μg of cell lysates (Fig. 3A, lane 2) and mAb B2 immunoprecipitates from 2.5 mg of cell lysates (Fig. 3A, lane 4) were comparable, Thus, approximately 2% of RAD51 was present in mAb B2 immunoprecipitates. When adjusted for the approximately 10% efficiency of BRCA2 immunoprecipitation by mAb B2 (Fig. 1C), I estimate that at least 10-20% of RAD51 was associated with BRCA2 in MCF7 cells.

The levels of both the mutant BRCA2 protein and RAD51 were substantially lower in CAPAN-1 cells (Fig. 1D, lane 1; Fig. 3A, lane 1) than those of the corresponding proteins in MCF7 cells (Fig. Fig. 1D, lane 2; Fig. 3A, lane 2). Thus, in order to compare the ability of the truncated BRCA2 protein to interact RAD51 with that of the wild type BRCA2 protein, I increased the amount of CAPAN-1 cell lysate used for co-immunoprecipitation to give similar levels of the proteins of interest to those in MCF7 cells (Fig. 3B, lanes 1 and 2). Under these conditions, the amount of RAD51 co-immunoprecipitated with the truncated BRCA2 in CAPAN-1 cells (Fig.3B, lane 3) was comparable to that observed with wild-type BRCA2 in MCF7 cells (Fig. 3B, lane 4). These findings are consistent with the in vitro mapping of RAD51 binding sites to each of the eight BRC motifs in human BRCA2 (36) retained by the mutant BRCA2 product in CAPAN-1 cells.

I also transfected 293T cells with pHA-RAD51, an N-terminal HA epitope tagged RAD51 construct. HA-RAD51 was then immunoprecipitated with anti-HA, and immunoblotted with anti-C15. As shown in Fig. 3C, BRCA2 was detected in HA-RAD51 immunoprecipitates from pHA-RAD51 but not the control vector transfected 293T cells. The presence of HA-RAD51 in BRCA2 immunoprecipitates was

demonstrated in pHA-RAD51 transfected 293T cells as well (Fig. 3C, bottom panel). As specificity controls, none of the proteins were detected in immunoprecipitates with anti-IgG under the same conditions (Fig. 3C, lane 5). All of these results establish that complexes containing BRCA2 and RAD51 occur in vivo.

**BRCA2 forms a complex with p53 in vivo.** BRCA2's nuclear localization and its putative transcriptional activation domain (32) suggest that this protein may associate with a transcriptional complex. Since p53-mediated cell cycle arrest is induced after DNA damage to allow DNA repair (37), I sought to investigate whether BRCA2 and p53 might be physically present in the same complex. To do so, endogenous p53 was immunoprecipitated from MCF7 cell lysates by monoclonal antibody DO-1, and immunoblotted with anti-C15. As shown in Fig. 4A, endogenous BRCA2 was readily detectable in p53-containing immunoprecipitates of MCF7 cells. In contrast, neither CAPAN-1, nor a p53 negative bladder cancer cell line, EJ, showed evidence of such immunocomplexes (Fig.4A, lanes 4 and 6). The IgG heavy chain has a gel mobility similar to p53, making it difficult to reliably detect p53 by immunoblotting after immunoprecipitation. In order to perform reciprocal experiments to determine whether endogenous p53 was present in BRCA2 immunoprecipitates, I labeled cells with $^{35}$S-methionine. BRCA2-containing complexes were first immunoprecipitated with anti-C15, eluted, and then immunoprecipitated with DO-1. As shown in Fig. 4B, a [$^{35}$S] labeled 53-kDa band was observed in autoradiographs of BRCA2/p53 double immunoprecipitates of MCF7, but not CAPAN-1 or EJ cells (Fig.4B, lanes 4 and 6). These findings establish that BRCA2 and p53 exist in vivo in the same complexes.

**BRCA2 inhibits p53 transcriptional activity.** To investigate possible functional interactions between BRCA2 and p53, I tested whether BRCA2 might act as a regulator for p53's transcriptional activity. To do so, MCF7 cells were transfected with pGFPB2 and PGluc, a luciferase reporter plasmid containing two copies of the p53-responsive element. As shown in Fig. 5, BRCA2 caused a decrease in the basal activation of p53-responsive elements by endogenous p53, when compared to transfection with the control vector pEGFPC1. BRCA2 also caused marked inhibition of exogenous p53 stimulation of PGluc. Co-transfection with BRCA2 had a similar inhibitory effect on exogenous p53-stimulated transcriptional activity in the osteosarcoma cell line, SAOS2, and the small cell lung carcinoma cell line, H1299 (data not shown). I found no difference in the level of p53 protein level expressed in cells in the presence or absence of co-transfection with BRCA2 (data not shown), excluding the possibility that inhibition by BRCA2 was the result of changes in p53 protein level. As a specificity control, GAL4luc, a luciferase reporter containing four copies of GAL4 responsive elements, was not inhibited by co-transfection of BRCA2 with GAL4-VP16, a vector expressing the GAL4 DNA binding domain fused to the transactivation domain of VP16 (data not shown). Thus, BRCA2 was not a general transcriptional inhibitor but appeared to specifically inhibit p53 transcriptional activity.

**RAD51 enhances BRCA2 inhibition of p53 transcriptional activity.** If the inhibitory effect of BRCA2 on p53's transcriptional activity reflects an intersection of cell cycle checkpoint and DNA repair pathways, I reasoned that RAD51 might affect p53's transcriptional activity as well. As shown in Fig. 5, RAD51 had no effect on the activation of p53-responsive elements by endogenous p53 in MCF7 cells or in response to exogenous p53. However, co-transfection of RAD51 with BRCA2 caused nearly complete inhibition of PGluc activation by either endogenous or exogenous p53. These results indicate that RAD51 enhances BRCA2 inhibition of p53's transcriptional activity.

## Discussion

Since BRCA2 is extremely large, in order to obtain antibodies with high specificity and sensitivity for characterizing it, I have generated five polyclonal antibodies against BRCA2 to provide good coverage for the entire molecule. Two of them were generated against peptides, and three others were generated against GST-fusion proteins (Fig. 1A). After careful characterization of these polyclonal antibodies, I

found that the two peptide antibodies (anti-N19 and anti-C15) and the antibody against GSTB3 region can specifically detect BRCA2 by Western blot, and anti-C15 is also capable of immunoprecipitating BRCA2 protein. However, the other two polyclonal antibodies (against GSTB1 and GSTB3) did not give a specific signal in these applications. None of these polyclonal antibodies was able to reliably detect endogenous BRCA2 by standard immunofluorescent methods. To obtain antibodies for all purposes, I generated and characterized three monoclonal antibodies against GSTB1, GSTB2 and GSTB3 respectively. While these antibodies work very well in immunoprecipitation and immunoblotting, they do not give a reliable pattern in immunofluorescent staining. To determine where BRCA2 is located within a cell, I constructed a GFP tagged *BRCA2* construct to localize the transfected GFP-BRCA2 protein in live 293T cells by fluorescence microscopy. Through these efforts, I identified the *BRCA2* gene product as a 460 kDa nuclear phosphoprotein.

Both BRCA2 full length mRNA and protein were found in all the cell lines examined except CAPAN-1 (Table 1). The expression level of BRCA2 was cell-cycle regulated both in normal and cancer cells. Thus, the alteration in BRCA2's expression level does not seem to correlate with tumorigenesis.

The identification of molecules potentially interacting with BRCA2 can provide clues about BRCA2's function. By using a yeast two-hybrid system to identify genes potentially interacting with BRCA2, I have identified RAD51. The in vivo complex containing BRCA2 and RAD51 was detected by co-immunoprecipitation. Recently Chen et al. (44) also reported detection of immunocomplexes containing BRCA2 and RAD51. My findings established that a major fraction of endogenous RAD51 is associated with the endogenous BRCA2 protein, implying that this interaction is functionally significant.

I demonstrated further that CAPAN-1 tumor cells, which contain a *BRCA2* mutation producing a truncated product, express a single BRCA2 immunoreactive species of around 230 kDa, consistent with the predicted size of the mutant. Of note, the mutant protein, though expressed at low level, interacted with RAD51 as efficiently as the wild type BRCA2. This mutant would be predicted to retain all eight repetitive BRC motifs which have been mapped in vitro to be RAD51 binding sites in human BRCA2 (36). These results point to a possible mechanism by which truncated BRCA2 mutants found commonly in tumors (Breast Cancer Information Core) may actually sequester RAD51 in a nonfunctional complex.

The p53 tumor suppressor gene is known to mediate cell cycle arrest following DNA damage (37) and there are reports that p53 can be detected in complexes with RAD51 (38, 39). I established here that p53 exists in *in vivo* complexes containing BRCA2. Moreover, a functional interaction between BRCA2 and p53 was demonstrated by evidence that BRCA2 inhibits p53 transcriptional activity. These results are consistent with the possibility that BRCA2 may act to limit the length or severity of p53-mediated cell cycle arrest following DNA damage. Other studies have shown that *Brca2* mutant mouse embryos exhibit a growth arrest phenotype (27-29, 33, 34), and that this phenotype is less severe in *Brca2/p53* double mutant mouse embryos (28). In addition, *Brca2* mutant embryo cells exhibit increased $p21^{WAF1/CIP1}$ levels associated with a defect in cell proliferation (29, 33, 34). All of these findings are consistent with a role of BRCA2 in down-regulating p53 transcriptional activity.

BRCA2, RAD51 and BRCA1 have similar patterns of expression (reviewed in ref. 45) and mouse mutants of each of these genes exhibit embryonic lethality (25-29, 46, 47) which can be partially rescued in a *p53* null background (28, 47, 48). These finding imply that all of these molecules have related functions. BRCA1 and BRCA2 have been reported to have transcriptional activity potential (30-32). Although RAD51 has been shown to be a component of the RNA polymerase II holoenzyme (49), it is not, itself, known to have transcriptional activity (reviewed in ref. 45). I did not detect any effect of RAD51 on p53 transcriptional activity. However, I did observe cooperative down-regulation of p53's activity by RAD51 and BRCA2, providing evidence that RAD51 is coupled to transcription pathways through its interactions with BRCA2 and p53. Thus, BRCA2 appears to serve as a regulator linking both cell cycle control and DNA repair pathways. It is likely that BRCA2's role in breast cancer may be due to

impaired or enhanced function of the BRCA2 gene product through abnormal interactions with other molecules with oncogenic potential.

In relation to the Statement of Work outlined in the proposal, I have completed Tasks 1-3, 5, 8-11; most of Tasks 4, 6, and 7; and part of Tasks 12 and 15.

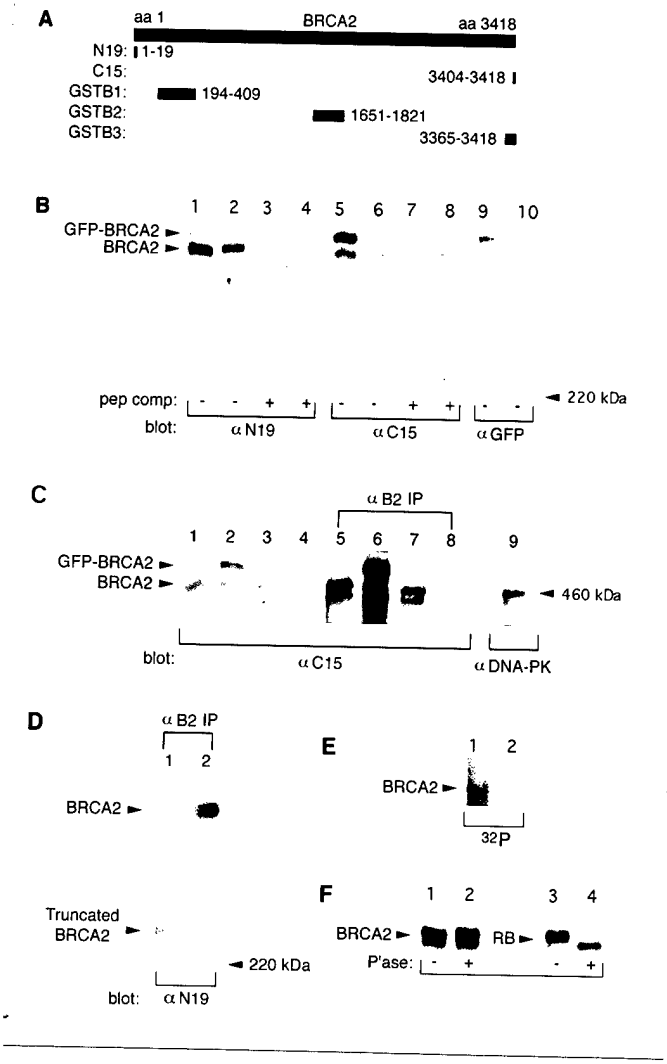


**Figure 1.** Characterization of BRCA2 protein. (A) Diagram showing the relative positions of peptides (N19 and C15) and GST-fusion proteins (GSTB1, GSTB2 and GSTB3) used to generate antibodies against BRCA2. (B) Lysates (50 μg of total protein) of 293T cells transfected with pGFPB2 (lanes 1, 3, 5, 7 and 9) or control vector pEGFPC1 (lanes 2, 4, 6, 8 and 10) were subjected to 4% SDS-PAGE, and immunoblotted with the indicated antibodies with (lanes 3, 4, 7, and 8) or without (lanes 1, 2, 5, 6, 9 and 10) homologous peptide competition. The GFP tag at the N-terminus of BRCA2 may interfere with recognition of BRCA2 by anti-N19 which is specific for the N-terminus of BRCA2, based on the relatively stronger signal for GFP-BRCA2 using anti-C15. (C) Cell lysates (50 μg of total protein; lanes 1 to 4), mAb B2 immunoprecipitates (from 1 mg of total protein; lanes 5 to 8) and DNA-PK immunoprecipitates (from 1 mg of total protein; lane 9) were resolved on the same 4% SDS-PAGE gel, and immunoblotted with anti-C15 or anti-DNA-PK. Cells included 293T transfected with pEGFPC1 (lanes 1 and 5) or pGFPB2 (lanes 2 and 6), MCF7 (lanes 3, 7 and 9), and CAPAN-1 (lanes 4 and 8). (D) mAb B2 immunprecipitates from 2 mg of total protein of CAPAN-1 (lane 1) and MCF7 (lane 2) were resolved by 5% SDS-PAGE, and immunoblotted with anti-N19. (E) MCF7 (lane 1) and CAPAN-1 (lane 2) cell lysates were labeled with [$^{32}$P] phosphoric acid, immunoprecipitated with mAb B2, resolved on a 4% SDS-PAGE, and exposed to Kodak X-Omat AR film. (F) BRCA2 or RB immunoprecipitates of MCF7 cells were untreated (lanes 1 and 3) or treated (lanes 2 and 4) with lambda-phosphatase, separated by 4% (BRCA2) or 8% (RB) SDS-PAGE, and immunoblotted with anti-C15 or anti-RB.

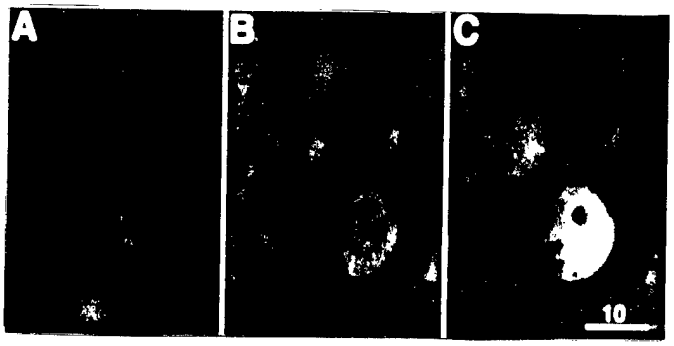


**Figure 2.** Subcellular localization of exogenously expressed BRCA2. (A)-(C) are representative images of live 293T cells transfected with pGFPB2. (A) Image showing the green signal of GFP-BRCA2 in transfected 293T cells. (B) Image showing the nuclei of cells in the same field labeled with Hoechst. Hoechst labeling is indicated as red color instead of its authentic blue color. (C) Overlay of the images in (A) and (B). Bar = 10 μm.

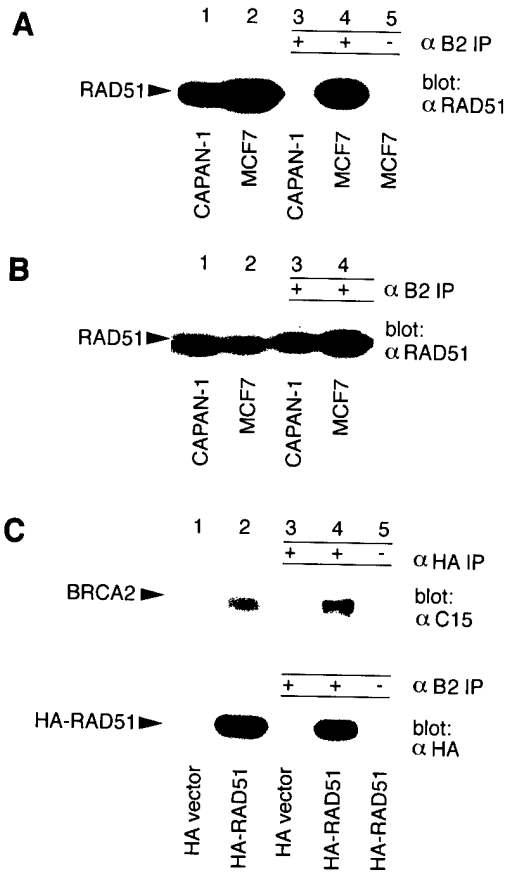


**Figure 3.** In vivo interactions of BRCA2 with RAD51. (A) Cell lysates (50 μg of total protein; lanes 1 and 2) and mAb B2 (lanes 3 and 4) or anti-IgG (lane 5) immunoprecipitates (from 2.5 mg of total protein) were resolved by 10% SDS-PAGE, and immunoblotted with anti-RAD51. (B) Cell lysates (50 μg of total protein for CAPAN-1, and 25 μg of total protein for MCF7; lanes 1 and 2) and mAb B2 immunoprecipitates (from 4 mg of total protein for CAPAN-1, and 2 mg of total protein for MCF7; lanes 3 and 4) were resolved by 10% SDS-PAGE, and immunoblotted with anti-RAD51. (C) 293T cells transfected with pHA-RAD51 or the control HA vector were lysed and immunoprecipitated with anti-HA, mAb B2 or anti-IgG. Lysates (lanes 1 and 2) and immunoprecipitates (lanes 3 to 5) were separated by 4% SDS-PAGE, and immunoblotted with anti-C15, or 10% SDS-PAGE, and immunoblotted with anti-HA. Anti-IgG immunoprecipitates from HA-RAD51 transfected cells were used as a specificity control (lane 5).

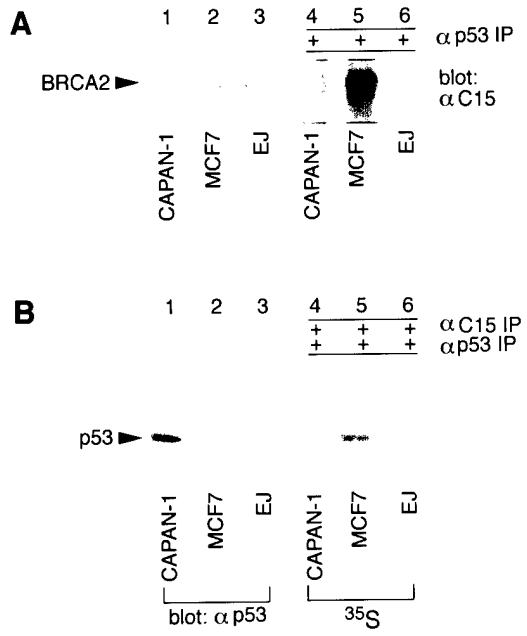


**Figure 4.** BRCA2 forms a complex with p53 in vivo. (A) Cell lysates (40 μg of total protein; lanes 1 to 3) and p53 immunoprecipitates (from 4 mg of total protein; lanes 4 to 6) were resolved by 6% SDS-PAGE, and immunoblotted with anti-C15. (B) Cell lysates (lanes 1 to 3) were separated by 10% SDS-PAGE, and immunoblotted with anti-p53 PAb421. Anti-C15 immunoprecipitates from $^{35}$S-methionine labeled cells were eluted, and re-precipitated with anti-p53 DO-1. The double immunoprecipitates were resolved by 10% SDS-PAGE and subjected to fluorography (lanes 4 to 6).

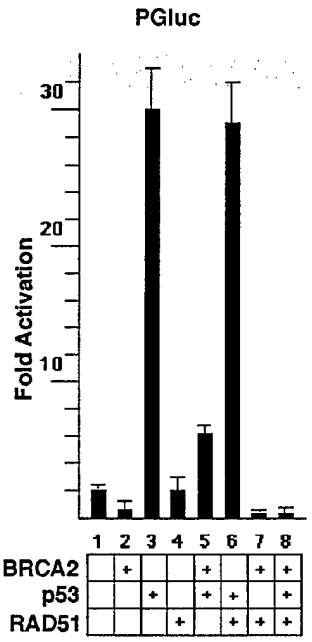


**Figure 5.** RAD51 enhances BRCA2 inhibition of p53's transcriptional activity. MCF cells were co-transfected with the indicated plasmids and PGluc. 48 hr. after transfection, luciferase activity was measured as described in Materials and Methods.

**Table 1.** BRCA2's expression in different cell lines

| Cell line | BRCA2 | Cell line | BRCA2 | Cell line | BRCA2 | Cell line | BRCA2 |
|---|---|---|---|---|---|---|---|
| SKBR3 | + | MDAMB453 | + | CAOV3 | + | HUTU80 | + |
| BT20 | + | MDAMB468 | + | PA-1 | + | DLD-1 | + |
| BT474 | + | MDAMB134VI | + | SKOV-3 | + | HEPG2 | + |
| MCF7 | + | ZR751 | + | SW626 | + | SW837 | + |
| MCF10A | + | ZR7530 | + | ME180 | + | BXPC3 | + |
| MDAMB157 | + | ED | + | HELAS3 | + | EJ | + |
| MDAMB175VII | + | MDAMB435S | + | MS751 | + | SW480 | + |
| MDAMB231 | + | AB5 | + | C4I | + | 293T | + |
| MDAMB361 | + | 129.NU2819 | + | C4II | + | CAPAN-1 | - |
| MDAMB415 | + | HS578T | + | AGS | + | CAPAN-2 | + |

Note: + stands for visible 11-kb/460-kDa band, - for the absence of a visible 11-kb/460-kDa band in Northern blot/Western blot.

# Conclusions

In this study, I identified the *BRCA2* gene product as a 460 kDa nuclear phosphoprotein which forms a complex with RAD51 in vivo. My findings established that a major fraction of endogenous RAD51 is associated with the endogenous BRCA2 protein, implying that this interaction is functionally significant.

I demonstrated further that CAPAN-1 tumor cells, which contain a *BRCA2* mutation producing a truncated product, express a single BRCA2 immunoreactive species of around 230 kDa, consistent with the predicted size of the mutant. Of note, the mutant protein, though expressed at low level, interacted with RAD51 as efficiently as the wild type BRCA2. This mutant would be predicted to retain all eight repetitive BRC motifs which have been mapped in vitro to be RAD51 binding sites in human BRCA2 (36). These results point to a possible mechanism by which truncated BRCA2 mutants found commonly in tumors (Breast Cancer Information Core) may actually sequester RAD51 in a nonfunctional complex.

I established that p53 exists in vivo in complexes containing BRCA2. Moreover, a functional interaction between BRCA2 and p53 was demonstrated by evidence that BRCA2 inhibits p53 transcriptional activity. These results are consistent with the possibility that BRCA2 may act to limit the length or severity of p53-mediated cell cycle arrest following DNA damage.

I did not detect any effect of RAD51 on p53 transcriptional activity. However, I did observe cooperative down-regulation of p53's activity by RAD51 and BRCA2, providing evidence that RAD51 is coupled to transcription pathways through its interactions with BRCA2 and p53. Thus, BRCA2 appears to serve as a regulator linking both cell cycle control and DNA repair pathways.

This study includes the first evidence of quantitative physiological interactions between endogenous BRCA2 and RAD51, the first demonstration of complexes and functional interactions involving BRCA2 and p53, the first evidence that BRCA2 is a phosphoprotein, and the first characterization of monoclonal antibodies with which to immunoprecipitate and characterize BRCA2. These antibodies should be valuable to the entire field. This initial characterization of the BRCA2 gene product provides a basis for further functional study on BRCA2. The demonstration of BRCA2's possible role in cell cycle control and DNA repair pathways opens up new directions in this research field.

# References


1. Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M., Ding, W. et al. *Science* **266**, 66-71 (1994).
2. Wooster, R., Neuhausen, S.L., Mangion, J., Quirk, Y.,Ford, D., Collins, N., Nguyen, K., Seal, S., Tran, T., Averill, D. et al. *Science* **265**, 2088-2090 (1994).
3. Wooster, R., Bignell, G., Lancaster, J., Swift, S., Seal, S., Mangion, J., Collins, N., Gregory, S., Gumbs, C., Micklem, G. et al. *Nature (London)* **378**, 789-792 (1995).
4. Tavtigian, S.V., Simard, J., Rommens, J., Couch, F., Shattuckeidens, D., Neuhausen, S., Merajver, S., Thorlacius, S., Offit, K., Stoppalyonnet, D. et al. *Nature Genet.* **12**, 333-337 (1996).
5. Futreal, P.A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, L.M., Haugen-Strano, A., Swensen, J., Miki, Y., et al. *Science* **266**, 120-122 (1994).
6. Friedman, L.S., Ostermeyer, E.A., Szabo, C.I., Dowd, P., Lynch, E.D., Rowell, S.E., and King, M.-C. *Nat. Genet.* **8**, 399-404 (1994).
7. Castilla, L.H., Couch, F.J., Erdos, M.R., Hoskins, K.F., Calzone, K., Garber, J.E., Boyd, J., Lubin, M.B.,Deshano, M.L., Brody, L.C., Collins, F.S., and Weber, B.L. *Nat. Genet.* **8**, 387-391 (1994).
8. Simard, J., Tonin, P., Durocher, F., Morgan, K., Rommens, J., Gingras, S., Samson, C., Leblanc, J.-F.,Belanger, C., Dion, F., et al. *Nat. Genet.* **8**, 392-398 (1994).
9. Phelan, C.M., Lancaster, J.M., Tonin, P., Gumbs, C., Cochran, C., Carter, R., Ghadirian, P., Perret, C., Moslehi, R., Dion, F., et al. *Nat. Genet.* **13**, 120-122 (1996).
10. Takahashi, H., Chiu, H.-C., Bandera, C.A., Behbakht, K., Liu, P.C., Couch, F.J., Weber, B.L., LiVolsi, V.A., Furusato, M., Rebane, B.A., et al. *Cancer Res.***56**, 2738-2741 (1996).
11. Claus, E.B., Risch, N., and Thompson, W.D. *Am. J. Hum. Genet.* **48**, 232-242 (1991).
12. Ford, D., Easton, D.F., Stratton, M., Narod, S., Goldgar, D., Devilee, P., Bishop, D.T., Weber, B., Lenoir, G., Chang-Claude, J., et al. *Am. J. Hum. Genet.* **62**, 676-689 (1998).
13. Lancaster, J.M., Wooster, R., Mangion, J., Phelan, C.M., Cochran, C., Gumbs, C., Seal, S., Barfoot, R., Collins, N., Bignell, G., et al. *Nat. Genet.* **13**, 238-240 (1996).
14. Miki, Y., Katagiri, T., Kasumi, F., Yoshimoto, T., and Nakamura, Y. *Nat. Genet.* **13**, 245-247 (1996).
15. Teng, D.H.-F., Bogden, R., Mitchell, J., Baumgard, M., Bell, R., Berry, S., Davis, T., Ha, P.C., Kehrer, R., Jammulapati, S., et al. *Nat. Genet.* **13**, 241-244 (1996).
16. Bork, P., Blomberg, N., and Nilges, M. *Nat. Genet.* **13**, 22-23 (1996).
17. Bignell, G., Micklem, G., Stratton, M.R., Ashworth, A., & Wooster, R. *Hum. Mol. Genet.* **6**, 53-58 (1997).
18. McAllister, K. A., Haugen-Strano, A., Hagevik, S., Brownlee, H.A., Collins, N.K., Futreal, P.A., Bennett, L.M. & Wiseman, R.W. *Cancer Res.* **57**, 3121-3125 (1997).
19. Rajan, J. V., Wang, M., Marquis, S.T. & Chodosh, L.A. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 13078-13083 (1996).
20. Vaughn, J. P., Cirisano, F.D., Huper, G., Berchuck, A., Futreal, P.A., Marks, J.R. & Igelhart, D. *Cancer Res.* **56**, 4590-4594 (1996).
21. Gudas, J.M., Li, T., Nguyen, H., Jensen, D., Rauscher III, F.J., and Cowan, K.H. *Cell Growth & Differ.* **7**, 717-723 (1996).
22. Lane, T.F., Deng, C., Elson, A., Lyu, M.S., Kozak, C.A. & Leder, P. *Genes & Develop,* **9**, 2712-2722 (1995).
23. Marquis, S.T., Rajan, J.V., Wynshaw-Boris, A., Xu, J., Yin, G-Y., Abel, K.J., Weber, B.L. & Chodosh, L.A. *Nat. Genet.* **11**, 1-26 (1995).

24. Rajan, J. V., Marquis, S.T., Gardner, H.P. & Chodosh, L.A. *Dev. Biol.* **184,** 385-401 (1997).
25. Gowen, L.C., Johnson, B.L., Latour, A.M., Sulik, K.K. & Koller, B.H. *Nature Genet.* **12**, 191-194 (1996).
26. Hakem, R., Pompa, J.L.D.L., Sirard, C., Mo, R., Woo, M., Hakem, A., Wakeham, A., Potter, J., Reitmair, A., Billia, F. et al. *Cell* **85**, 1009-1023 (1996).
27. Sharan, S. K., Morimatsu, M., Albrecht, U., Lim, D-S., Regel, E., Dinh, C., Sands, A., Eichele, G., Hasty, P. & Bradley, A. *Nature (London)* **386**, 804-810 (1997).
28. Ludwig, T., Chapman, D.L., Papaioannou, V.E. & Efstratiadis, A. *Genes & Dev.* **11**, 1226-1241 (1997).
29. Suzuki, A., de la Pompa, J.L., Hakem, R., Elia, A., Yoshida, R., Mo, R., Nishina, H., Chuang, T., Wakeham, A., Itie, A., Koo, W., Billia, P., Ho, A., Fukumoto, M., Hui, C.C. & Mak, T.W. *Genes & Dev.* **11**, 1242-1252 (1997).
30. Chapman, M. S. & Verma, I.M. *Nature* **382,** 678-679 (1996).
31. Monteiro, A. N., August, A. & Hanafusa, H. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13595-13599 (1996).
32. Milner, J., Ponder, B., Hughes-Davies, L., Seltmann, M. & Kouzarides, T. *Nature (London)* **386**, 772-773 (1997).
33. Connor, F., Bertwistle, D., Mee, P.J., Ross, G.M., Swift, S., Grigorieva, E., Tybulewicz, V.L.J. & Ashworth, A. *Nature Genet.* **17**, 423-430 (1997).
34. Patel, K.J., Yu, V.P.C.C., Lee, H., Corcoran, A., Thistlethwaite, f.C., Evans, M.J., Colledge, W.H., Friedman, L.S., Ponder, B.A.J. & Venkitaraman, A.R. *Mol. Cell* **1**, 347-357 (1998).
35. Mizuta, R., LaSalle, J.M., Cheng, H-L., Shinohara, A., Ogawa, H., Copeland, N., Jenkins, N.A., LaLande, M. & Alt, F.W. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 6927-6932 (1997).
36. Wong, A.K.C., Pero, R., Ormonde, P.A., Tavtigian, S.V.& Bartel, P.L. *J. Biol. Chem.* **272**, 31941-31944 (1997).
37. Levine, A.J. *Cell* **88**,323-331 (1997).
38. Stürzbecher, H.-W., Donzelmann, B., Henning, W., Knippschild, U. & Buchhop, S. *EMBO J.* **15**, 1992-2002 (1996).
39. Buchhop, S., Gibson, M.K., Wang, X.W., Wagner, P., Stürzbecher, H.-W., Donzelmann, B., Henning, W., Knippschild, U. & Harris, C.C. *Nucleic Acids Res.* **19**, 3868-3874 (1997).
40. Scully, R., Chen, J., Ochs, R.L., Keegan, K., Hoekstra, M., Feunteun, J. & Livingston, D. M. *Cell* **90**, 1-20 (1997).
41. Goggins, M., Schutte, M., Lu, J., Moskaluk, C.A., Weinstein, C.L., Petersen, G.M., Yeo, C.J., Jackson, C.E., Lynch, H.T., Hruban, R.H. & Kern, S.E. *Cancer Res.* **56**, 5360-5364 (1996).
42. Sherr, C.J. *Trends in Cell Biol.* **4**, 15-18 (1994).
43. Bertwistle, D., Swift, S., Marston, N.J., Jackson, L.E., Crossland, S., Crompton, M.R., Marshall, C.J. & Ashworth, A. *Cancer Res.* **57**, 5485-5488 (1997).
44. Chen, P.-L., Chen, C.-F., Chen, Y., Xiao, J., Sharp, Z.D. & Lee, W.-H. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 5287-5292 (1998).
45. Zhang, H., Tombline, G. & Weber, B.L. *Cell* **92**, 433-436 (1998).
46. Tsuzuki, T. et al. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 6236-6240 (1996).
47. Lim, D.-S. & Hasty, P. *Mol. Cell. Biol.* **16**, 7133-7143 (1996).
48. Hakem, R., Pompa, J.L.D.L., Elia, A., Potter, J. & Mak, T.W. *Nature Genet.* **16**, 298-302 (1997).
49. Maldonado, E., Shiekhattar, R., Sheldon, M., Cho, H., Drapkin, R., Rickert, P., Lees, E., Anderson, C.W., Linn, S. & Reinberg, D. *Nature (London)* **381**, 86-89 (1996).

# Appendices

Copies of an abstract and a journal article resulting from the work funded by this grant are attached:

1. Abstract:
   Marmorstein, L.Y., Ouchi, T., Bartel, P. and Aaronson, S. A. (1998). The nuclear phosphoprotein BRCA2 inhibits p53 activity synergistically with RAD51. Pathways to cancer, 30. (Cold Spring Harbor Laboratory).

2. Journal article:
   Marmorstein, L.Y., Ouchi, T., and Aaronson, S. A. The *BRCA2* gene product functionally interacts with p53 and RAD51. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 13869-13874.

# THE NUCLEAR PHOSPHOPROTEIN BRCA2 INHIBITS P53 ACTIVITY SYNERGISTICALLY WITH RAD51

Lihua Y. Marmorstein[1], Toru Ouchi[1], Paul Bartel[2] and Stuart A. Aaronson[1]

[1]The Derald H. Ruttenberg Cancer Center, The Mount Sinai Medical Center, One Gustave L. Levy Place, New York, NY 10029; [2]Myriad Genetics, Inc., Salt Lake City, Utah 84108

Germline mutations in the human BRCA2 gene confer susceptibility to early-onset familial breast cancers. Mouse embryonic fibroblasts lacking BRCA2 are defective in proliferation presumably mediated by over-expression of p53 and $p21^{WAF1/CIP1}$. These cells are also hypersensitive to radiation and defective in DNA repair. BRCA2 was shown to interact with RAD51 in a two-hybrid system. p53 was also shown to interact with RAD51 and inhibit RAD51's ATPase activity. However, it is not yet known how BRCA2 may be involved in p53 checkpoint and RAD51 DNA repair pathways. To facilitate the study of BRCA2 function, we generated a series of polyclonal and monoclonal antibodies specific for the BRCA2 protein. Our data indicates that the BRCA2 gene product is a 450-kDa phosphoprotein. Moreover, live cell image analysis of 293T cells transiently transfected with a GFP tagged BRCA2 full-length cDNA construct revealed that BRCA2 protein is localized to the cell nucleus.

By using a luciferase reporter assay, we show that transfection of BRCA2 into breast cancer cells expressing wild-type p53 inhibits p53 transcriptional activity, and suppresses exogenous p53 activation of p53-responsive elements. In addition, co-transfection of BRCA2 and RAD51 synergistically inhibits p53's activity. By using the antibodies generated, we demonstrated that endogenous BRCA2 can be co-immunoprecipitated in complexes containing either p53 or RAD51. These results suggest that BRCA2 functionally and physically interacts with these key components of cell cycle control and DNA repair pathways.

Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 13869–13874, November 1998
Medical Sciences

# The *BRCA2* gene product functionally interacts with p53 and RAD51

LIHUA Y. MARMORSTEIN, TORU OUCHI, AND STUART A. AARONSON*

The Derald H. Ruttenberg Cancer Center, The Mount Sinai Medical Center, One Gustave L. Levy Place, New York, NY 10029

*Communicated by Leon A. Heppel, Cornell University, Ithaca, NY, September 21, 1998 (received for review July 10, 1998)*

ABSTRACT **Germ-line mutations in the human *BRCA2* gene confer susceptibility to breast cancer. Efforts to elucidate its function have revealed a putative transcriptional activation domain and *in vitro* interaction with the DNA repair protein RAD51. Other studies have indicated that RAD51 physically associates with the p53 tumor suppressor protein. Here we show that the *BRCA2* gene product is a 460-kDa nuclear phosphoprotein, which forms *in vivo* complexes with both p53 and RAD51. Moreover, exogenous BRCA2 expression in cancer cells inhibits p53's transcriptional activity, and RAD51 coexpression enhances BRCA2's inhibitory effects. These findings demonstrate that BRCA2 physically and functionally interacts with two key components of cell cycle control and DNA repair pathways. Thus, BRCA2 likely participates with p53 and RAD51 in maintaining genome integrity.**

Germ-line mutations in the human *BRCA1* and *BRCA2* breast cancer suppressor genes confer susceptibility to breast and ovarian cancers (1–4). Mutations in *BRCA1* and *BRCA2* are believed to be responsible for most hereditary breast cancers (3–10), which account for 5–10% of all breast cancer cases (11). About 52% of the families with four or more breast cancer cases have inherited mutations in *BRCA1*, and 32% possess *BRCA2* mutations (12). In contrast, somatic mutations in *BRCA1* and *BRCA2* are rare in sporadic cases of breast cancer (5, 13–15). Both BRCA1 and BRCA2 are up-regulated in proliferating and differentiating cells, expressed in a cell cycle-dependent manner peaking at the G1/S boundary (19–21), widely expressed during development (22–24), and essential for early embryonic development (25–29). Recent evidence indicates that BRCA1 is involved in transcriptional regulation (30, 31).

The *BRCA2* gene is composed of 27 exons and encodes a predicted 384-kDa protein possessing no obvious homology to other sequences in publicly available databases (4). There are eight repetitive units in the BRCA2 protein sequence (BRC motifs; refs. 16 and 17) and a potential nuclear localization signal within its C terminus (18). BRCA2 also possesses a putative transcriptional activation domain in exon 3, suggesting a role of BRCA2 in the regulation of gene expression (32). Evidence for a possible function of BRCA2 in DNA repair has been suggested by recent findings that Brca2 mutant mouse embryos are defective in DNA repair (27, 33, 34). Moreover, yeast two-hybrid and glutathione *S*-transferase (GST) pull-down analysis revealed that BRCA2 interacts *in vitro* with RAD51, a protein involved in DNA double-strand break repair and homologous recombination (27, 35, 36). Binding sites for RAD51 have been mapped to each of the eight BRC motifs in human BRCA2 (36) and to a C-terminal region of mouse Brca2 (27, 35).

Structural and functional characterization of the endogenous BRCA2 protein have been hampered by the large size of the protein and the lack of suitable immunological reagents for its detection. In the present studies, we generated polyclonal and monoclonal antibodies to characterize endogenous BRCA2 and identify proteins that form complexes with this protein. We demonstrate that BRCA2 is a nuclear phosphoprotein that associates *in vivo* with a significant portion of the endogenous pool of RAD51. Because an immediate cellular reponse to DNA damage is p53-mediated cell cycle arrest (37), and RAD51 has been reported to physically associate with p53 (38, 39), we also investigated whether a physical and functional relationship could be detected between BRCA2 and p53.

## MATERIALS AND METHODS

**Generation of BRCA2 Antibodies.** Polyclonal antibodies were raised in rabbits, and mAbs were generated in mice. The peptides were conjugated to keyhole limpet hemacyanin. The three GST-fusion regions were generated by reverse transcription–PCR amplification using the following primers: 5′-GGTCAAGTTCTTTAGCTACAGGATCCACCC-3′ and 5′-CTCCATCTGGGCTCCATGTCGACCTGAAAG-3′ for GSTB1, 5′-CCTGCAACTTGTTACAGAATTCAGTCCCC-3′ and 5′-AGGGTGAAGAGCTAGTCTCGAGTTCCTCAA-3′ for GSTB2, and 5′-GTCAGTGAATCCACTAGAATTCCTCCCACC-3′ and 5′-TGGTCTTGAACTCCTGGCCTCGAGCACT-3′ for GSTB3. These regions then were subcloned into the GST-fusion protein expression vector pGEX-5X-2 or pGEX-4T-2 (Pharmacia) and verified by sequencing. All antibodies were affinity-purified.

**Plasmid Constructs.** pGFPB2 containing human full-length *BRCA2* cDNA fused in-frame with green fluorescent protein (GFP) was constructed as follows. A fragment at the N terminus of *BRCA2* was generated from pUCBRCA2 by PCR amplification using a high-fidelity PCR system (Boehringer Mennheim) and the following primers: 5′-GGCCAGATCTATGCCTATTGGATCCAAAGAGAGG-3′ and 5′-GGCCGTCGACTGCTTGTTTATCACCTGTGTCTCC-3′. This fragment was subcloned into the pEGFPC1 vector (CLONTECH) at *Bgl*II and *Sal*I sites to generate pGFPB2N. A *Sse*8387I–*Sal*I *BRCA2* fragment released from pUCBRCA2 then was ligated with pGFPB2N digested with *Bgl*II and *Sse*8387I to generate pGFPB2. Sequencing confirmed the identity of the fragment generated by PCR. A human full-length RAD51 cDNA with *Bam*HI and *Sal*I flanking was amplified from a human testis cDNA library (CLONTECH) by using *Pfu* DNA polymerase (Stratagene) and the following primers: 5′-GGCCGGATCCATGGCAATGCAGATGCAGCTTG-3′ and 5′-GGCCGTCGACTCTTTGGCATCTCCCACTCCAT-3′. Cloning of this cDNA fragment into pcDNA3HA generated an N-terminal hemagglutinin (HA)-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.



Abbreviations: GST, glutathione *S*-transferase; GFP, green fluorescent protein; HA, hemagglutinin; RB, retinoblastoma protein.

*To whom reprint requests should be addressed. e-mail: Aaronson@smtplink.mssm.edu.

tagged human Rad51 construct, pHA-RAD51. A *Bgl*II–*Bam*HI fragment containing two copies of the consensus p53 binding sequence and the adenovirus major late TATA box from p50–2 (a gift from A. J. Levine, Princeton Univ., Princeton, NJ) were cloned into the pGL2 vector (Promega) to generate a luciferase reporter plasmid PGluc. GAL4luc and GAL4-VP16 were a gift from R. Davis (Univ. of Massachusetts, Worcester).

**Fluorescence Microscopy.** pGFPB2 was transiently transfected into 293T cells in 6-well plates containing coverslips using Lipofectamine (GIBCO). Twenty-four hours after transfection, cells were incubated with Hoechst stain (bisBenzimide Trihydrochloride Hoechst No. 33258; 5 $\mu$g/ml; Sigma) for 15 min to label nuclei. Coverslips were transferred to a Sykes-Moore perfusion chamber (Belco) mounted on the stage of a Nikon Eclipse E600 microscope and maintained at 37°C. Images were collected by using a Nikon Plan Apo ×100 oil immersion objective lens (numerical aperture = 1.4), a 1,000 × 800 pixel back-illuminated cooled charge-coupled device camera (Princeton Instruments, Trenton, NJ), and Metamorph software (Universal Imaging, Media, PA).

**Luciferase Assays.** Plasmid DNA was transiently transfected into cells by using Lipofectamine PLUS (GIBCO). Approximately $2 \times 10^6$ cells were cotransfected with 1 $\mu$g of reporter plasmid and 1 $\mu$g of effector plasmid except for pGFPB2 (5 $\mu$g). Cells were harvested 48 hr after transfection, and luciferase activity was measured by using a luciferase assay kit (Promega). The transfection efficiency was normalized by measuring $\beta$-galactosidase activities after the cotransfection of MFG-$\beta$-galactosidase plasmid.

**Immunoprecipitation and Immunoblotting.** Cell lysates were prepared in lysis buffer (50 mM Tris·HCl, pH 8.0/120 mM NaCl/0.5% Nonidet P-40/10 $\mu$g/ml aprotinin/10 $\mu$g/ml leupeptin/1 mM phenylmethylsulfonyl fluoride/1 mM sodium orthovanadate). In some cases, cells were labeled with [$^{35}$S]methionine (0.1 mCi/ml) or [$^{32}$P]phosphoric acid (0.25 mCi/ml; NEN) for 3 hr. The total protein content of the lysates was determined by bicinchoninic acid assay (Pierce). Phosphatase treatment of immunoprecipitates was performed as described (40). Cell lysates or immunoprecipitates were electrophoresed on 4%, 6%, or 10% SDS-polyacrylamide gels and transferred onto a poly(vinylidene difluoride) membrane (Millipore). ECL or ECL Plus (Amersham) was used for signal detection. Anti-GFP antibody was obtained from CLONTECH, antibodies against p53 (PAb421), DNA-dependent protein kinase, or RAD51 from Calbiochem, antibodies against the HA epitope or p53 (DO-1) from Santa Cruz Biotechnology, and anti-retinoblastoma protein (RB) antibody from PharMingen.

## RESULTS

**Characterization of the *BRCA2* Gene Product.** To facilitate the study of BRCA2 structure and function, we generated a series of polyclonal and monoclonal antibodies by using both peptides and GST fusion proteins corresponding to various regions of the molecule (Fig. 1*A*). On Western blots of 293T cell lysates, affinity-purified polyclonal antibodies against both the N-terminal (anti-N19) and C-terminal peptides (anti-C15) recognized a band at a molecular mass much greater than that of the 220-kDa myosin standard (Fig. 1*B*). This band also was detected in MCF7 cells (see Fig. 3*C*, lane 3) and comigrated with 460-kDa DNA-dependent protein kinase (Fig. 1*C*, lane 9). However, it was not detected by anti-C15 in CAPAN-1 cells (Fig. 1*C*, lane 4), a human pancreatic tumor cell line that has lost one *BRCA2* allele and contains the 6174delT mutation in the remaining allele (41).

To confirm that this band reflected the authentic BRCA2 protein, we transfected 293T cells with the plasmid pGFPB2 containing full-length *BRCA2* cDNA fused at its N terminus to GFP or with the control vector pEGFPC1. Both anti-N19 and anti-C15 specifically recognized a band in pGFPB2-transfected, but not in vector-transfected, 293T cells at the predicted size of the GFP-BRCA2 fusion protein, approxi-

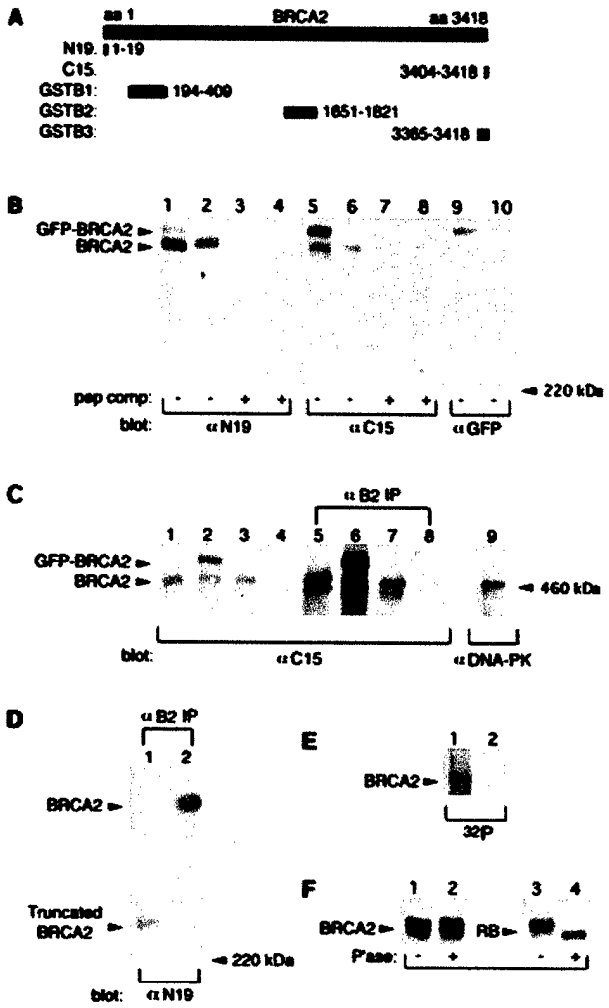


FIG. 1. Characterization of BRCA2 protein. (*A*) Diagram showing the relative positions of peptides (N19 and C15) and GST-fusion proteins (GSTB1, GSTB2, and GSTB3) used to generate antibodies against BRCA2. Among antibodies generated against GST-fusion proteins, we selected a mAb generated against GSTB2 for use in this study. (*B*) Lysates (50 $\mu$g of total protein) of 293T cells transfected with pGFPB2 (lanes 1, 3, 5, 7, and 9) or control vector pEGFPC1 (lanes 2, 4, 6, 8, and 10) were subjected to 4% SDS/PAGE, and immunoblotted with the indicated antibodies with (lanes 3, 4, 7, and 8) or without (lanes 1, 2, 5, 6, 9, and 10) homologous peptide competition. The GFP tag at the N terminus of BRCA2 may interfere with recognition of BRCA2 by anti-N19, which is specific for the N terminus of BRCA2, based on the relatively stronger signal for GFP-BRCA2 using anti-C15. (*C*) Cell lysates (50 $\mu$g of total protein; lanes 1–4), mAb B2 immunoprecipitates (from 1 mg of total protein; lanes 5–8), and DNA-dependent protein kinase immunoprecipitates (1 mg of total protein; lane 9) were resolved on the same 4% SDS/PAGE gel, and immunoblotted with anti-C15 or anti-DNA-protein kinase. Cells included 293T transfected with pEGFPC1 (lanes 1 and 5) or pGFPB2 (lanes 2 and 6), MCF7 (lanes 3, 7, and 9), and CAPAN-1 (lanes 4 and 8). (*D*) mAb B2 immunprecipitates from 2 mg of total protein of CAPAN-1 (lane 1) and MCF7 (lane 2) were resolved by 5% SDS/PAGE and immunoblotted with anti-N19. (*E*) MCF7 (lane 1) and CAPAN-1 (lane 2) cell lysates were labeled with [$^{32}$P]phosphoric acid, immunoprecipitated with mAb B2, resolved on a 4% SDS/PAGE, and exposed to Kodak X-Omat AR film. (*F*) BRCA2 or RB immunoprecipitates of MCF7 cells were untreated (lanes 1 and 3) or treated (lanes 2 and 4) with lambda-phosphatase, separated by 4% (BRCA2) or 8% (RB) SDS/PAGE, and immunoblotted with anti-C15 or anti-RB.

mately 27 kDa higher in mass than the native BRCA2 protein (Fig. 1*B*, lanes 1 and 5). A protein of the same size also was recognized by a polyclonal antibody against GFP (Fig. 1*B*, lane 9), confirming its identity as GFP-BRCA2. Moreover, all of these bands were specifically competed by the homologous peptides (Fig. 1*B*). Further confirmation that the proteins we had identified represent the authentic BRCA2 protein was provided by immunoprecipitation/Western blot experiments. MCF7, CAPAN-1, or transfected 293T cell lysates were immunoprecipitated with a mAb (mAb B2) raised against the GSTB2 region of BRCA2 (Fig. 1*A*), and then immunoblotted with anti-C15. The expected 460-kDa BRCA2 protein was detected in MCF7 and 293T cells but not in CAPAN-1 cells, and the 487-kDa GFP-BRCA2 fusion protein was detected only in pGFPB2-transfected 293T cells (Fig. 1*C*). All of these findings firmly established the identity of the 460-kDa protein as the product of the *BRCA2* gene.

When BRCA2 was immunoprecipitated with mAb B2 from CAPAN-1 or MCF cell lysates and immunoblotted with anti-N19, a truncated BRCA2 protein was specifically detected in CAPAN-1 cells (Fig. 1*D*, lane 1). This truncated species migrated above the 220-kDa myosin standard and had an estimated molecular mass of 230 kDa, nearly matching the predicted size of 224 kDa. These results indicate that the mutated *BRCA2* gene in CAPAN-1 cells encodes a stable truncated protein.

It should be noted that both anti-C15 and mAb B2 recognized a BRCA2 doublet in MCF7 and 293T cells (Fig. 1 *B* and *C*), suggesting the possibility of posttranslational processing, such as phosphorylation. Evidence that BRCA2 was phosphorylated derived from [$^{32}$P]-labeling experiments, in which a [$^{32}$P]-labeled band at 460 kDa was detected in autoradiographs of mAb B2 immunoprecipitates of MCF7, but not CAPAN-1 cells (Fig. 1*E*). However, when mAb B2 immunoprecipitates from MCF7 cells were treated with lambda-phosphatase, the BRCA2 doublet was not altered. Under the same conditions, the RB doublet, which is known to result from phosphorylation (42), was reduced to a single band (Fig. 1*F*). Thus, phosphorylation was unlikely to account for the BRCA2 doublet. It also should be noted that anti-N19 recognized only the upper band of the BRCA2 doublet, and that GFP-BRCA2 migrated as a single band as well (Fig. 1 *B* and *C*). Thus, the doublet likely resulted from BRCA2 amino-terminal processing or as an alternative product of the *BRCA2* gene.

**Exogenously Expressed BRCA2 Localizes to the Nucleus.** BRCA2 reportedly possesses a potential nuclear localization signal in its C terminus (18), and recently Bertwistle *et al.* (43) reported that BRCA2 was enriched in the nuclear fraction derived from MCF7 cells. Although our antibodies recognized GFP-BRCA2 overexpressed in transfected cells, it was not possible to reliably detect endogenous BRCA2 by standard immunofluorescent methods (unpublished data). However, the availability of the GFP-tagged *BRCA2* construct made it possible to localize the transfected GFP-BRCA2 protein in live 293T cells by fluorescence microscopy. Although GFP alone was distributed throughout the cell (data not shown), GFP-BRCA2 primarily was localized to the Hoechst-labeled nuclei (Fig. 2). These results suggest a nuclear localization of the BRCA2 protein.

***In vivo* Interaction of BRCA2 with RAD51.** BRCA2 has been reported to interact with RAD51 *in vitro* (27, 35, 36). To investigate whether these proteins form a complex *in vivo*, BRCA2 was immunoprecipitated with mAb B2 from MCF7 or CAPAN-1 cell lysates and immunoblotted with anti-RAD51. As shown in Fig. 3*A*, endogenous RAD51 was readily detected in a complex with endogenous BRCA2 in MCF7 cells (Fig. 3*A*, lane 4), whereas there was a faint signal for RAD51 coimmunoprecipitated with the truncated BRCA2 protein in CAPAN-1 cells (Fig. 3*A*, lane 3). As a specificity control, anti-IgG

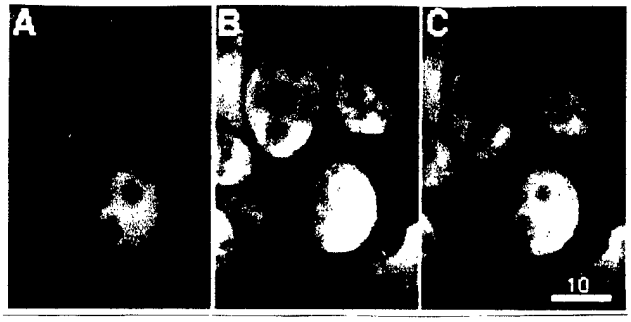


FIG. 2. Subcellular localization of exogenously expressed BRCA2. Representative images of live 293T cells transfected with pGFPB2. (*A*) Image showing the green signal of GFP-BRCA2 in transfected 293T cells. (*B*) Image showing the nuclei of cells in the same field labeled with Hoechst. Hoechst labeling is indicated as red color instead of its authentic blue color. (*C*) Overlay of the images in *A* and *B*. (Bar = 10 $\mu$m.)

failed to immunoprecipitate complexes containing RAD51 from MCF7 cells (Fig. 3*A*, lane 5). The signal intensities for RAD51 in 50 $\mu$g of cell lysates (Fig. 3*A*, lane 2) and mAb B2 immunoprecipitates from 2.5 mg of cell lysates (Fig. 3*A*, lane 4) were comparable. Thus, approximately 2% of RAD51 was present in mAb B2 immunoprecipitates. When adjusted for the approximately 10% efficiency of BRCA2 immunoprecipita-

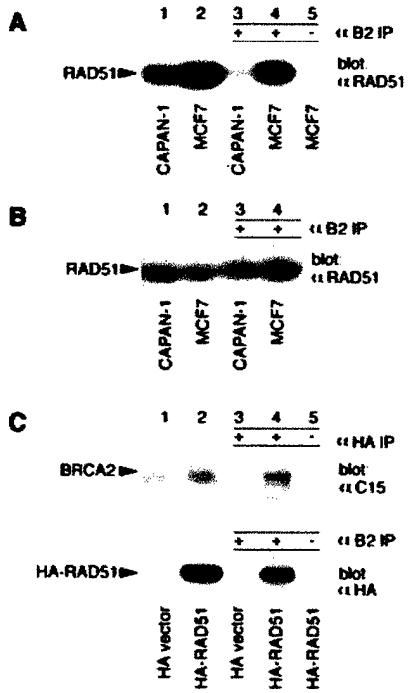


FIG. 3. *In vivo* interactions of BRCA2 with RAD51. (*A*) Cell lysates (50 $\mu$g of total protein; lanes 1 and 2) and mAb B2 (lanes 3 and 4) or anti-IgG (lane 5) immunoprecipitates (from 2.5 mg of total protein) were resolved by 10% SDS/PAGE, and immunoblotted with anti-RAD51. (*B*) Cell lysates (50 $\mu$g of total protein for CAPAN-1, and 25 $\mu$g of total protein for MCF7; lanes 1 and 2) and mAb B2 immunoprecipitates (from 4 mg of total protein for CAPAN-1, and 2 mg of total protein for MCF7; lanes 3 and 4) were resolved by 10% SDS/PAGE and immunoblotted with anti-RAD51. (*C*) 293T cells transfected with pHA-RAD51 or the control HA vector were lysed and immunoprecipitated with anti-HA, mAb B2, or anti-IgG. Lysates (lanes 1 and 2) and immunoprecipitates (lanes 3–5) were separated by 4% SDS/PAGE and immunoblotted with anti-C15, or 10% SDS/PAGE and immunoblotted with anti-HA. Anti-IgG immunoprecipitates from HA-RAD51 transfected cells were used as a specificity control (lane 5).

tion by mAb B2 (Fig. 1*C*), we estimate that at least 10–20% of RAD51 was associated with BRCA2 in MCF7 cells.

The levels of both the mutant BRCA2 protein and RAD51 were substantially lower in CAPAN-1 cells (Fig. 1*D*, lane 1; Fig. 3*A*, lane 1) than those of the corresponding proteins in MCF7 cells (Fig. 1*D*, lane 2; Fig. 3*A*, lane 2). Thus, to compare the ability of the truncated BRCA2 protein to interact RAD51 with that of the wild-type BRCA2 protein, we increased the amount of CAPAN-1 cell lysate used for coimmunoprecipitation to give similar levels of the proteins of interest to those in MCF7 cells (Fig. 3*B*, lanes 1 and 2). Under these conditions, the amount of RAD51 coimmunoprecipitated with the truncated BRCA2 in CAPAN-1 cells (Fig. 3*B*, lane 3) was comparable to that observed with wild-type BRCA2 in MCF7 cells (Fig. 3*B*, lane 4). These findings are consistent with the *in vitro* mapping of RAD51 binding sites to each of the eight BRC motifs in human BRCA2 (36) retained by the mutant BRCA2 product in CAPAN-1 cells.

We also transfected 293T cells with pHA-RAD51, an N-terminal HA epitope-tagged RAD51 construct. HA-RAD51 then was immunoprecipitated with anti-HA and immunoblotted with anti-C15. As shown in Fig. 3*C*, BRCA2 was detected in HA-RAD51 immunoprecipitates from pHA-RAD51 but not the control vector-transfected 293T cells. The presence of HA-RAD51 in BRCA2 immunoprecipitates was demonstrated in pHA-RAD51-transfected 293T cells as well (Fig. 3*C*, *Lower*). As specificity controls, none of the proteins were detected in immunoprecipitates with anti-IgG under the same conditions (Fig. 3*C*, lane 5). All of these results establish that complexes containing BRCA2 and RAD51 occur *in vivo*.

**BRCA2 Forms a Complex with p53 *in vivo*.** BRCA2's nuclear localization and its putative transcriptional activation domain (32) suggest that this protein may associate with a transcriptional complex. Because p53-mediated cell cycle arrest is induced after DNA damage to allow DNA repair (37), we sought to investigate whether BRCA2 and p53 might be physically present in the same complex. To do so, endogenous p53 was immunoprecipitated from MCF7 cell lysates by mAb DO-1 and immunoblotted with anti-C15. As shown in Fig. 4*A*, endogenous BRCA2 was readily detectable in p53-containing immunoprecipitates of MCF7 cells. In contrast, neither CAPAN-1 nor a p53 negative bladder cancer cell line, EJ, showed evidence of such immunocomplexes (Fig. 4*A*, lanes 4 and 6). The IgG heavy chain has a gel mobility similar to p53, making it difficult to reliably detect p53 by immunoblotting after immunoprecipitation. To perform reciprocal experiments to determine whether endogenous p53 was present in BRCA2 immunoprecipitates, we labeled cells with [$^{35}$S]methionine. BRCA2-containing complexes first were immunoprecipitated with anti-C15, eluted, and then immunoprecipitated with DO-1. As shown in Fig. 4*B*, a [$^{35}$S]-labeled 53-kDa band was observed in autoradiographs of BRCA2/p53 double immunoprecipitates of MCF7, but not CAPAN-1 or EJ cells (Fig. 4*B*, lanes 4 and 6). These findings establish that BRCA2 and p53 exist *in vivo* in the same complexes.

**BRCA2 Inhibits p53 Transcriptional Activity.** To investigate possible functional interactions between BRCA2 and p53, we tested whether BRCA2 might act as a regulator for p53's transcriptional activity. To do so, MCF7 cells were transfected with pGFPB2 and PGluc, a luciferase reporter plasmid containing two copies of the p53-responsive element. As shown in Fig. 5, BRCA2 caused a decrease in the basal activation of p53-responsive elements by endogenous p53, when compared with transfection with the control vector pEGFPC1. BRCA2 also caused marked inhibition of exogenous p53 stimulation of PGluc. Cotransfection with BRCA2 had a similar inhibitory effect on exogenous p53-stimulated transcriptional activity in the osteosarcoma cell line, SAOS2, and the small cell lung carcinoma cell line, H1299 (data not shown). We found no difference in the level of p53 protein level expressed in cells in the presence or absence of cotransfection with BRCA2 (data not shown), excluding the possibility that inhibition by BRCA2 was the result of changes in p53 protein level. As a specificity control, GAL4luc, a luciferase reporter containing four copies of GAL4 responsive elements, was not inhibited by cotransfection of BRCA2 with GAL4-VP16, a vector expressing the GAL4 DNA binding domain fused to the transactivation domain of VP16 (data not shown). Thus, BRCA2 was not a general transcriptional inhibitor but appeared to specifically inhibit p53 transcriptional activity.

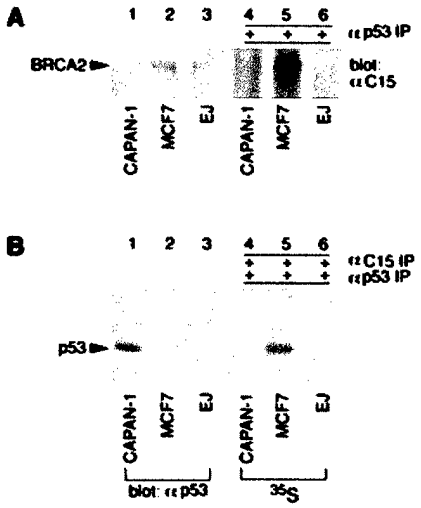


FIG. 4. BRCA2 forms a complex with p53 *in vivo*. (*A*) Cell lysates (40 μg of total protein; lanes 1–3) and p53 immunoprecipitates (4 mg of total protein; lanes 4–6) were resolved by 6% SDS/PAGE and immunoblotted with anti-C15. (*B*) Cell lysates (lanes 1–3) were separated by 10% SDS/PAGE and immunoblotted with anti-p53 PAb421. Anti-C15 immunoprecipitates from [$^{35}$S]-methionine-labeled cells were eluted and reprecipitated with anti-p53 DO-1. The double immunoprecipitates were resolved by 10% SDS/PAGE and subjected to fluorography (lanes 4–6).

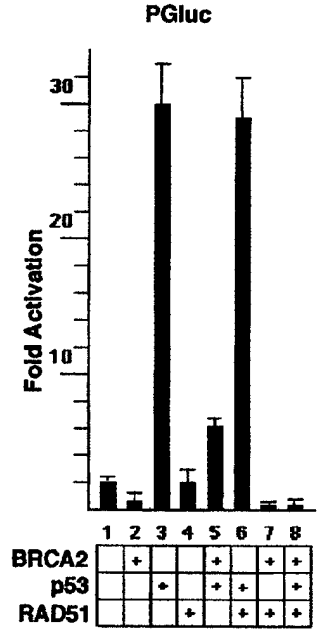


FIG. 5. RAD51 enhances BRCA2 inhibition of p53's transcriptional activity. MCF7 cells were cotransfected with the indicated plasmids and PGluc. Forty-eight hours after transfection, luciferase activity was measured as described in *Materials and Methods*.

**RAD51 Enhances BRCA2 Inhibition of p53 Transcriptional Activity.** If the inhibitory effect of BRCA2 on p53's transcriptional activity reflects an intersection of cell cycle checkpoint and DNA repair pathways, we reasoned that RAD51 might affect p53's transcriptional activity as well. As shown in Fig. 5, RAD51 had no effect on the activation of p53-responsive elements by endogenous p53 in MCF7 cells or in response to exogenous p53. However, cotransfection of RAD51 with BRCA2 caused nearly complete inhibition of PGluc activation by either endogenous or exogenous p53. These results indicate that RAD51 enhances BRCA2 inhibition of p53's transcriptional activity.

## DISCUSSION

Abnormalities caused by targeted disruption of the *Brca2* gene include increased sensitivity to DNA damage induced by ionizing irradiation, UV light, and other genotoxic agents (27, 33, 34). The accumulation of double-strand DNA breaks and chromosomal abnormalities combined with the lack of obvious checkpoint or apoptotic response abnormalities in *Brca2* mutant cells have implied a role of BRCA2 in DNA repair (33, 34). Recent findings that BRCA2 and RAD51 interact *in vitro* have suggested further that BRCA2 may be involved in RAD51-mediated repair pathways (27, 35, 36). In this study, we identified the *BRCA2* gene product as a 460-kDa nuclear phosphoprotein that forms a complex with RAD51 *in vivo*. While this manuscript was in preparation, Chen *et al.* (44) reported detection of BRCA2 as a nuclear protein, consistent with our findings. They also reported detection of immunocomplexes containing BRCA2 and RAD51 (44). Our findings established that a major fraction of endogenous RAD51 is associated with the endogenous BRCA2 protein, implying that this interaction is functionally significant.

We demonstrated further that CAPAN-1 tumor cells, which contain a *BRCA2* mutation producing a truncated product, express a single BRCA2 immunoreactive species of around 230 kDa, consistent with the predicted size of the mutant. Of note, the mutant protein, though expressed at low level, interacted with RAD51 as efficiently as the wild-type BRCA2. This mutant would be predicted to retain all eight repetitive BRC motifs that have been mapped *in vitro* to be RAD51 binding sites in human BRCA2 (36). These results point to a possible mechanism by which truncated BRCA2 mutants found commonly in tumors (Breast Cancer Information Core) actually may sequester RAD51 in a nonfunctional complex.

The p53 tumor suppressor gene is known to mediate cell cycle arrest after DNA damage (37), and there are reports that p53 can be detected in complexes with RAD51 (38, 39). We established here that p53 exists in *in vivo* complexes containing BRCA2. Moreover, a functional interaction between BRCA2 and p53 was demonstrated by evidence that BRCA2 inhibits p53 transcriptional activity. These results are consistent with the possibility that BRCA2 may act to limit the length or severity of p53-mediated cell cycle arrest after DNA damage. Other studies have shown that *Brca2* mutant mouse embryos exhibit a growth arrest phenotype (27–29, 33, 34), and that this phenotype is less severe in *Brca2/p53* double mutant mouse embryos (28). In addition, *Brca2* mutant embryo cells exhibit increased $p21^{WAF1/CIP1}$ levels associated with a defect in cell proliferation (29, 33, 34). All of these findings are consistent with a role of BRCA2 in down-regulating p53 transcriptional activity.

*BRCA2*, *RAD51*, and *BRCA1* have similar patterns of expression (45), and mouse mutants of each of these genes exhibit embryonic lethality (25–29, 46, 47) that can be partially rescued in a *p53* null background (28, 47, 48). These findings imply that all of these molecules have related functions. BRCA1 and BRCA2 have been reported to have transcriptional activity potential (30–32). Although RAD51 has been shown to be a component of the RNA polymerase II holoenzyme (49), it is not itself known to have transcriptional activity (45). We did not detect any effect of RAD51 on p53 transcriptional activity. However, we did observe cooperative down-regulation of p53's activity by RAD51 and BRCA2, providing evidence that RAD51 is coupled to transcription pathways through its interactions with BRCA2 and p53. Thus, BRCA2 appears to serve as a regulator linking both cell cycle control and DNA repair pathways.

We are indebted to Dr. S. V. Tavtigian, Dr. A. K. C. Wong, and Dr. Paul Bartel for the full-length *BRCA2* cDNA, and Dr. Alan D. Marmorstein for advice and assistance on fluorescence microscopy. We also thank Ms. Mutsuko Ouchi for technical assistance and the members of the Aaronson Laboratory for helpful discussion. This work was supported by National Institutes of Health Specialized Program of Research Excellence in breast cancer 1P50CA68425 (S.A.A.) and an Incentive Award from Mount Sinai School of Medicine (T.O.). L.Y.M. is a recipient of Postdoctoral Fellowship DAMD17-97-1-7317 from the Department of the Army.

1. Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P. A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L. M., Ding, W., *et al.* (1994) *Science* **266**, 66–71.
2. Wooster, R., Neuhausen, S. L., Mangion, J., Quirk, Y., Ford, D., Collins, N., Nguyen, K., Seal, S., Tran, T., Averill, D., *et al.* (1994) *Science* **265**, 2088–2090.
3. Wooster, R., Bignell, G., Lancaster, J., Swift, S., Seal, S., Mangion, J., Collins, N., Gregory, S., Gumbs, C., Micklem, G., *et al.* (1995) *Nature (London)* **378**, 789–792.
4. Tavtigian, S. V., Simard, J., Rommens, J., Couch, F., Shattuck-eidens, D., Neuhausen, S., Merajver, S., Thorlacius, S., Offit, K., Stoppalyonnet, D., *et al.* (1996) *Nat. Genet.* **12**, 333–337.
5. Futreal, P. A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, L. M., Haugen-Strano, A., Swensen, J., Miki, Y., *et al.* (1994) *Science* **266**, 120–122.
6. Friedman, L. S., Ostermeyer, E. A., Szabo, C. I., Dowd, P., Lynch, E. D., Rowell, S. E. & King, M.-C. (1994) *Nat. Genet.* **8**, 399–404.
7. Castilla, L. H., Couch, F. J., Erdos, M. R., Hoskins, K. F., Calzone, K., Garber, J. E., Boyd, J., Lubin, M. B., Deshano, M. L., Brody, L. C., *et al.* (1994) *Nat. Genet.* **8**, 387–391.
8. Simard, J., Tonin, P., Durocher, F., Morgan, K., Rommens, J., Gingras, S., Samson, C., Leblanc, J.-F., Belanger, C., Dion, F., *et al.* (1994) *Nat. Genet.* **8**, 392–398.
9. Phelan, C. M., Lancaster, J. M., Tonin, P., Gumbs, C., Cochran, C., Carter, R., Ghadirian, P., Perret, C., Moslehi, R., Dion, F., *et al.* (1996) *Nat. Genet.* **13**, 120–122.
10. Takahashi, H., Chiu, H.-C., Bandera, C. A., Behbakht, K., Liu, P. C., Couch, F. J., Weber, B. L., LiVolsi, V. A., Furusato, M., Rebane, B. A., *et al.* (1996) *Cancer Res.* **56**, 2738–2741.
11. Claus, E. B., Risch, N. & Thompson, W. D. (1991) *Am. J. Hum. Genet.* **48**, 232–242.
12. Ford, D., Easton, D. F., Stratton, M., Narod, S., Goldgar, D., Devilee, P., Bishop, D. T., Weber, B., Lenoir, G., Chang-Claude, J., *et al.* (1998) *Am. J. Hum. Genet.* **62**, 676–689.
13. Lancaster, J. M., Wooster, R., Mangion, J., Phelan, C. M., Cochran, C., Gumbs, C., Seal, S., Barfoot, R., Collins, N., Bignell, G., *et al.* (1996) *Nat. Genet.* **13**, 238–240.
14. Miki, Y., Katagiri, T., Kasumi, F., Yoshimoto, T. & Nakamura, Y. (1996) *Nat. Genet.* **13**, 245–247.
15. Teng, D. H.-F., Bogden, R., Mitchell, J., Baumgard, M., Bell, R., Berry, S., Davis, T., Ha, P. C., Kehrer, R., Jammulapati, S., *et al.* (1996) *Nat. Genet.* **13**, 241–244.
16. Bork, P., Blomberg, N. & Nilges, M. (1996) *Nat. Genet.* **13**, 22–23.
17. Bignell, G., Micklem, G., Stratton, M. R., Ashworth, A. & Wooster, R. (1997) *Hum. Mol. Genet.* **6**, 53–58.
18. McAllister, K. A., Haugen-Strano, A., Hagevik, S., Brownlee, H. A., Collins, N. K., Futreal, P. A., Bennett, L. M. & Wiseman, R. W. (1997) *Cancer Res.* **57**, 3121–3125.
19. Rajan, J. V., Wang, M., Marquis, S. T. & Chodosh, L. A. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 13078–13083.
20. Vaughn, J. P., Cirisano, F. D., Huper, G., Berchuck, A., Futreal, P. A., Marks, J. R. & Igelhart, D. (1996) *Cancer Res.* **56**, 4590–4594.
21. Gudas, J. M., Li, T., Nguyen, H., Jensen, D., Rauscher III, F. J. & Cowan, K. H. (1996) *Cell Growth Differ.* **7**, 717–723.

22. Lane, T. F., Deng, C., Elson, A., Lyu, M. S., Kozak, C. A. & Leder, P. (1995) *Genes Dev.* **9**, 2712–2722.
23. Marquis, S. T., Rajan, J. V., Wynshaw-Boris, A., Xu, J., Yin, G.-Y., Abel, K. J., Weber, B. L. & Chodosh, L. A. (1995) *Nat. Genet.* **11**, 1–26.
24. Rajan, J. V., Marquis, S. T., Gardner, H. P. & Chodosh, L. A. (1997) *Dev. Biol.* **184**, 385–401.
25. Gowen, L. C., Johnson, B. L., Latour, A. M., Sulik, K. K. & Koller, B. H. (1996) *Nat. Genet.* **12**, 191–194.
26. Hakem, R., Pompa, J. L. D. L., Sirard, C., Mo, R., Woo, M., Hakem, A., Wakeham, A., Potter, J., Reitmair, A., Billia, F., *et al.* (1996) *Cell* **85**, 1009–1023.
27. Sharan, S. K., Morimatsu, M., Albrecht, U., Lim, D.-S., Regel, E., Dinh, C., Sands, A., Eichele, G., Hasty, P. & Bradley, A. (1997) *Nature (London)* **386**, 804–810.
28. Ludwig, T., Chapman, D. L., Papaioannou, V. E. & Efstratiadis, A. (1997) *Genes Dev.* **11**, 1226–1241.
29. Suzuki, A., de la Pompa, J. L., Hakem, R., Elia, A., Yoshida, R., Mo, R., Nishina, H., Chuang, T., Wakeham, A., Itie, A., *et al.* (1997) *Genes Dev.* **11**, 1242–1252.
30. Chapman, M. S. & Verma, I. M. (1996) *Nature (London)* **382**, 678–679.
31. Monteiro, A. N., August, A. & Hanafusa, H. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 13595–13599.
32. Milner, J., Ponder, B., Hughes-Davies, L., Seltmann, M. & Kouzarides, T. (1997) *Nature (London)* **386**, 772–773.
33. Connor, F., Bertwistle, D., Mee, P. J., Ross, G. M., Swift, S., Grigorieva, E., Tybulewicz, V. L. J. & Ashworth, A. (1997) *Nat. Genet.* **17**, 423–430.
34. Patel, K. J., Yu, V. P. C. C., Lee, H., Corcoran, A., Thistlethwaite, F. C., Evans, M. J., Colledge, W. H., Friedman, L. S., Ponder, B. A. J. & Venkitaraman, A. R. (1998) *Mol. Cell* **1**, 347–357.
35. Mizuta, R., LaSalle, J. M., Cheng, H.-L., Shinohara, A., Ogawa, H., Copeland, N., Jenkins, N. A., LaLande, M. & Alt, F. W. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 6927–6932.
36. Wong, A. K. C., Pero, R., Ormonde, P. A., Tavtigian, S. V. & Bartel, P. L. (1997) *J. Biol. Chem.* **272**, 31941–31944.
37. Levine, A. J. (1997) *Cell* **88**, 323–331.
38. Stürzbecher, H.-W., Donzelmann, B., Henning, W., Knippschild, U. & Buchhop, S. (1996) *EMBO J.* **15**, 1992–2002.
39. Buchhop, S., Gibson, M. K., Wang, X. W., Wagner, P., Stürzbecher, H.-W., Donzelmann, B., Henning, W., Knippschild, U. & Harris, C. C. (1997) *Nucleic Acids Res.* **19**, 3868–3874.
40. Scully, R., Chen, J., Ochs, R. L., Keegan, K., Hoekstra, M., Feunteun, J. & Livingston, D. M. (1997) *Cell* **90**, 1–20.
41. Goggins, M., Schutte, M., Lu, J., Moskaluk, C. A., Weinstein, C. L., Petersen, G. M., Yeo, C. J., Jackson, C. E., Lynch, H. T., Hruban, R. H. & Kern, S. E. (1996) *Cancer Res.* **56**, 5360–5364.
42. Sherr, C. J. (1994) *Trends Cell Biol.* **4**, 15–18.
43. Bertwistle, D., Swift, S., Marston, N. J., Jackson, L. E., Crossland, S., Crompton, M. R., Marshall, C. J. & Ashworth, A. (1997) *Cancer Res.* **57**, 5485–5488.
44. Chen, P.-L., Chen, C.-F., Chen, Y., Xiao, J., Sharp, Z. D. & Lee, W.-H. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 5287–5292.
45. Zhang, H., Tombline, G. & Weber, B. L. (1998) *Cell* **92**, 433–436.
46. Tsuzuki, T., Fujii, Y., Sakumi, K., Tominaga, Y., Nakao, K., Sekiguchi, M., Matsushiro, A., Yoshimura, Y. & Morita, T. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 6236–6240.
47. Lim, D.-S. & Hasty, P. (1996) *Mol. Cell. Biol.* **16**, 7133–7143.
48. Hakem, R., Pompa, J. L. D. L., Elia, A., Potter, J. & Mak, T. W. (1997) *Nat. Genet.* **16**, 298–302.
49. Maldonado, E., Shieklattar, R., Sheldon, M., Cho, H., Drapkin, R., Rickert, P., Lees, E., Anderson, C. W., Linn, S. & Reinberg, D. (1996) *Nature (London)* **381**, 86–89.